

The Use of Spermatozoal Ultrastructure in Phylogenetic Studies of Tubificidae (Oligochaeta)

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ABSTRACT

The influence of patterns of spermatozoal ultrastructure on hypotheses of phylogenetic relationships within the Tubificidae is examined on the basis of knowledge for species representing 15 different genera. A parsimony analysis of a combination of spermatozoal and conventional morphological characters supports that the Phallodrilinae, Limnodriloidinae and Tubificinae are monophyletic taxa, and that the Rhyacodrilinae is a paraphyletic group as currently defined. The mature spermatozoa of *Heterodrilus* spp., *Pectinodrilus molestus*, *Coralliodrilus rugosus*, *Smithsonidrilus hummelincki* and *Tubificoides amplivasatus* are described for the first time.

RÉSUMÉ

L'utilisation de l'ultrastructure des spermatozoïdes pour les études phylogéniques sur les Tubificidae (Oligochaeta)

L'influence des différentes organisations ultrastructurales des spermatozoïdes sur les hypothèses concernant les relations phylétiques à l'intérieur des Tubificidae est examinée, à partir de nos connaissances sur des espèces représentant quinze genres différents. Une analyse de parcimonie portant sur une combinaison de caractères des spermatozoïdes et de la morphologie conventionnelle indique que les Phallodrilinae, Limnodriloidinae et Tubificinae sont des taxons monophylétiques, et que les Rhyacodrilinae dans leur définition actuelle sont un groupe paraphylétique. Les spermatozoïdes mûrs de *Heterodrilus* spp., *Pectinodrilus molestus*, *Coralliodrilus rugosus*, *Smithsonidrilus hummelincki* and *Tubificoides amplivasatus* sont décrits pour la première fois.

The ultrastructure of spermatozoa has proved useful for phylogenetic assessment of higher taxa within the Clitellata (=Euclitellata *sensu* Jamieson [33]), particularly with regard to family level relationships in oligochaetes [34-35, 37, 40]. Spermatozoa are fairly uniform and distinctive within several clitellate groups [41], and their more general appearance supports a close relationship between clitellates and onychophorans [36, 37]. On the other hand, their ultrastructure may sometimes be used to distinguish species within the same genus [27, 44]. Evidence for great variation in the sperm ultrastructure of Tubificidae, a speciose group of aquatic oligochaetes, has been accumulated in recent years [24].

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The spermatozoa of tubificids, as well as those of all other clitellates, are characterized by a sequence of acrosome, nucleus, middle piece and tail (Fig. 2e). The acrosome contains an acrosome tube involving the other acrosome structures. The middle piece contains only the mitochondria. In the basal body region of the tail there is a prominent basal cylinder from which the two central tubules of the axoneme start. The axoneme shows a 9+2 arrangement and is characterized by the presence of some sort of accessory structure of the central apparatus [21], and a peripheral ring of glycogen granules. In members of the subfamily Tubificinae a double sperm line produces euspermatozoa and paraspermatozoa. (These different sperm categories have been called "typical" and "atypical", respectively, by one of us [2, 23], but here we adopt the terminology of HEALY & JAMIESON [30].)

Conventional morphological characters of tubificids, as well as of other aquatic oligochaetes, are few and many similarities are due to homoplasy, i.e., convergence or reversal [3, 13, 15]. This means that the support for monophyly of some groups, e.g. some tubificid subfamilies, is weak. Additional data, structural as well as molecular, are needed for a better understanding of the phylogenetic relationships within the Tubificidae.

In the present paper, parsimony analyses of both spermatozoal and conventional characters of 15 tubificid genera (Table 1), representing four subfamilies, are presented. The two sets of characters were run separately and in combination, to examine different impacts on the phylogenetic hypotheses. The majority of the spermatozoal data are from the literature, but for six marine species (representing the genera *Heterodrilus*, *Pectinodrilus*, *Coralliodrilus*, *Smithsonidrilus*, *Tubificoides*) the spermatozoa are described for the first time. When euspermatozoa as well as paraspermatozoa are present, both types are described, but in the parsimony analyses only euspermatozoa are considered.

MATERIAL AND METHODS

The material of *Tubificoides amplivasatus* was collected in muddy sediments in the Oresund, Denmark, in the summer of 1982. Specimens of *Heterodrilus pentcheffi* and *H. minisetosus*, and a single individual of *Pectinodrilus molestus* were found in subtidal sand at Long Key, Florida Keys, Florida, in October 1992. Additional material of *P. molestus*, and specimens of *Coralliodrilus rugosus* and *Smithsonidrilus hummelincki* were collected at subtidal and intertidal sites near Carrie Bow Cay, on the barrier reef off Belize in Central America, in March 1993. The material of *T. amplivasatus* was fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The other material was fixed in a picric acid-glutaraldehyde-paraformaldehyde mixture following ERMAK & EAKIN [5]. After washing in the buffer and postfixation in similarly buffered 1% osmium tetroxide, the worms were dehydrated in a graded ethanol series and embedded in SPURR's resin. Sections were cut with LKB III and V, and observed under a JEOL 100XS electron microscope.

FITCH parsimony [25] analyses (i.e. multistate characters unordered) of tubificid taxa were performed using PAUP for Macintosh [43]. The branch-and-bound option (addition sequence: furthest) was selected. For analyses resulting in more than one equally parsimonious tree, strict consensus trees were calculated.

RESULTS

Description of new sperm types

Spermatozoa of Heterodrilus minisetosus and H. pentcheffi (Rhyacodrilinae) (Figs 1b, g, 1, 2c). Spermatozoa were examined in the spermathecae. The two species have similar spermatozoa and will thus be described together. Only euspermatozoa are present, and spermatozeugmata are not formed. In both species, the acrosome is straight, about 0.6 μm long, 0.1 μm wide, with the vesicle, acrosome rod and secondary tube all withdrawn. The nucleus is apically straight, basally loosely twisted, and is followed by five twisted mitochondria, 1.4 μm long, 0.4 μm wide. The tail has two tetragon fibres, one much longer than the other.

Spermatozoa of Pectinodrilus molestus (Phallodrilinae) (Figs 1c, f, 2e). Spermatozoa were examined in the spermathecae. Only euspermatozoa are present, and spermatozeugmata are not formed. The acrosome is straight, 0.5 μm long, 0.1 μm wide. A secondary tube (if present at all)

is not visible. The nucleus is apically corkscrew-shaped, then gradually becomes twisted and is basally almost straight. Five twisted mitochondria follow, 2.5 μm long, 0.36 μm wide. The tail has tetragon fibres.

Spermatozoa of Coralliodrilus rugosus (Phalldrilineae) (Figs 1a, k, o, 2d). Spermatozoa were examined in the spermathecae. Only euspermatozoa are present and spermatozeugmata are not formed. The acrosome tube is corkscrew-shaped with an helical ridge making 1.5 gyres. The acrosome rod is indistinct. A secondary tube is not present. The nucleus is apically corkscrew-shaped and follows the pitch of the acrosome tube, then becomes twisted with a pitch increasing towards the base. The five mitochondria (1.2 μm long, 0.3 μm wide) are always surrounded by residual cytoplasm. The tail has tetragon fibres.

Spermatozoa of Smithsonidrilus hummelincki (Limnodriloidinae) (Figs 1d-e, h, j, n, 2a-b). Spermatozoa were examined at the ciliated male funnels as well as in the spermathecae. Euspermatozoa and paraspermatozoa are constantly present and easily distinguished. At the funnels the two types are randomly mixed, whereas in the spermathecae they are grouped in different spermatozeugmata, each containing only one sperm type. Euspermatozoa have an acrosome, 0.7 μm long, 0.15 μm wide, formed by a thin-walled, straight acrosome tube with a limen, a distinct secondary tube, a short rod and a partly withdrawn acrosome vesicle. The nucleus is apically twisted and basally straight. Four to five subspherical mitochondria form the middle piece. The tail shows a prominent central sheath. The paraspermatozoa have a shorter (0.36 μm) acrosome with the vesicle completely external to the tube, but with no other structure; a thin and irregularly outlined nucleus; two to four mitochondria characteristically swollen when the sperm are in the spermatheca, but not when at the funnels; a tail with a swollen plasma membrane when the sperm are at the funnels, but with apparently degenerating axonemes when in the spermatheca. The paraspermatozoa are fewer than the euspermatozoa.

Spermatozoa of Tubificoides amplivasatus (Tubificinae) (Fig. 1i, m). Spermatozoa at the funnels as well as in the spermathecae were examined. Both euspermatozoa and paraspermatozoa are constantly present. Spermatozeugmata are found in the spermathecae. They are composed of the two sperm types grouped together in the typical tubificine way [22]. We have no data on the acrosome of the euspermatozoa. The nucleus is apically twisted and basally straight. Three small ovoidal mitochondria separate the nucleus from the tail which has, at least in part, a prominent central sheath. The paraspermatozoa show a straight, empty acrosome tube, 0.5 μm long, 0.1 μm wide, with a small acrosome vesicle completely external to it. The nucleus is rectilinear, 2-3 μm long (which is shorter than that of the euspermatozoa). It has the shape of an elongated cone and is partly uncondensed. The two mitochondria are longer and larger than those of the euspermatozoa. The tail has a plasma membrane widely separated from the axoneme.

Parsimony Analyses

Taxa. Sperm ultrastructure has been reasonably well studied for 17 species of Tubificidae, representing 15 genera (Table 1). These 15 "genera", as characterized by their representatives studied, are regarded as the ingroup taxa. The outgroup is an hypothetical ancestor, the spermatozoal characters of which are in accordance with the plesiomorphic model suggested by JAMIESON *et al.* [40]. For the morphological characters, the ancestor is coded as a member the Phreodrilidae, a putative sister group of the Tubificidae [15].

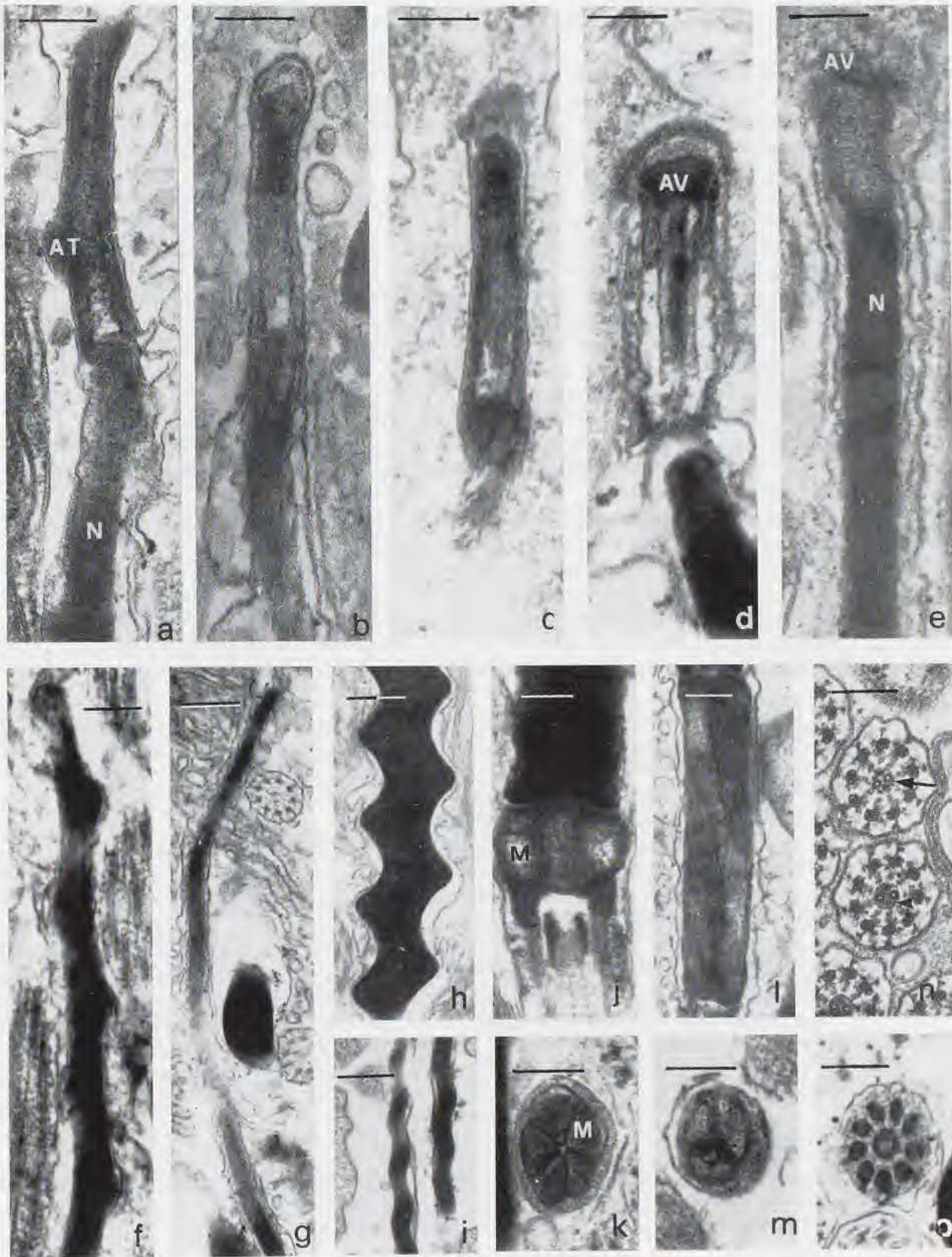
Characters and character states. Two sets of characters are used, referred to as the "spermatozoal" and "morphological characters", respectively. All multistate characters are treated as unordered. The character states of all taxa are coded in Table 1. The character states are identical for *Heterodrilus pencheffi* and *H. minisetosus*, and for *Inanidrilus leukodermatus* and *I. bulbosus*.

TABLE 1. — List of ingroup taxa included in the parsimony analyses. The genera are grouped according to their current subfamilial positions. ¹ *Rhyacodrilus arthingtonae* is an enigmatic species, possibly not a natural member of *Rhyacodrilus*, but it does not belong to *Rhizodrilus* [19] as was suggested before [1, 24].

Taxon	Characters 1-20	Reference
Ancestor (hypothetical)	00000 00000 00000 00000	[15, 40]
Rhyacodrilinae		
<i>Rhyacodrilus</i> ¹ <i>arthingtonae</i> Jamieson	00000 01000 ??111 00000	[32, 39]
<i>Rhizodrilus russus</i> Erséus	10110 11200 00111 00000	[16, 24]
<i>Monopylephorus limosus</i> (Hatai)	00010 11111 00101 00010	[20, 24]
<i>Heterodrilus pentcheffi</i> Erséus	00010 01010 11111 00010	[present study, 10]
<i>Heterodrilus minisetosus</i> Erséus		[present study, 10]
Phallogodrilinae		
<i>Bathydrilus formosus</i> Erséus	10010 11210 01110 00310	[present study, 12]
<i>Olavius planus</i> (Erséus)	00010 11210 01110 10310	[8, 24]
<i>Inanidrilus leukodermatus</i> (Giere)	00010 11210 01110 10310	[24, 28]
<i>Inanidrilus bulbosus</i> Erséus		[9, 24]
<i>Thalassodrilus prostates</i> (Knöllner)	01100 10110 12110 00310	[6, 24]
<i>Pectinodrilus molestus</i> (Erséus)	00010 11210 11110 00310	[present study, 14, 18]
<i>Coralliodrilus rugosus</i> Erséus	01010 11210 11110 00410	[present study, 17]
Limnodriloidinae		
<i>Smithsonidrilus hummelincki</i> (Righi & Kanner)	20011 01100 02100 01200	[11, 42]
<i>Thalassodrilides ineri</i> (Righi & Kanner)	10111 00010 01100 01200	[22, 42]
Tubificinae:		
<i>Tubifex tubifex</i> Müller	30010 01111 01000 00101	[2, 31]
<i>Clitellio arenarius</i> (Müller)	30010 01100 02100 00411	[24, 29]
<i>Tubificoides amplivasatus</i> (Erséus)	3???? ?101 02000 00101	[present study, 7, 24]

FIG. 1. — Character variation in tubificid spermatozoa (for interpretation of ultrastructural details, see Fig. 2). **a:** *Coralliodrilus rugosus*, longitudinal section of acrosome, bar 0.15 µm. **b:** *Heterodrilus minisetosus*, longitudinal section of acrosome, bar 0.15 µm. **c:** *Pectinodrilus molestus*, longitudinal section of acrosome, bar 0.15 µm. **d-e:** *Smithsonidrilus hummelincki*, longitudinal section of eusperm acrosome (d), and of nuclear tip and acrosome of paraspermatozoon (e), both bars 0.15 µm. **f:** *Pectinodrilus molestus*, corkscrew-shaped portion of nucleus, bar 0.2 µm. **g:** *Heterodrilus minisetosus*, apical, straight portion of nucleus, bar 0.2 µm. **h:** *Smithsonidrilus hummelincki*, twisted portion of eusperm nucleus, bar 0.3 µm. **i:** *Tubificoides amplivasatus*, twisted portion of two eusperm nuclei, bar 0.5 µm. **j:** *Smithsonidrilus hummelincki*, euspermatozoon, nuclear base (top), round mitochondria (centre), and basal body area with evident basal cylinder (bottom), bar 0.2 µm. **k:** *Coralliodrilus rugosus*, cross section of midpiece with five mitochondria (some residual cytoplasm present in the mature spermatozoon), bar 0.2 µm. **l:** *Heterodrilus minisetosus*, longitudinal section of midpiece, bar 0.2 µm. **m:** *Tubificoides amplivasatus*, cross section of midpiece with three mitochondria, bar 0.2 µm. **n:** *Smithsonidrilus hummelincki* euspermatozoon, cross section of tails at different levels, showing central apparatus with tetragon fibres (top), and prominent central sheath (bottom), bar 0.2 µm. **o:** *Coralliodrilus rugosus*, cross section of basal body area with basal cylinder, bar 0.2 µm.

Abbreviations in Figs 1 and 2: AR, acrosome rod; AT, acrosome tube; AV, acrosome vesicle; BC, basal cylinder; M, mitochondria in midpiece; N, nucleus; T, tail.



Spermatozoal characters

1. *Spermatozeugmata*: absent (0); present with only one type of spermatozoa (1); of two types present, one type formed by euspermatozoa, the other by paraspermatozoa (2); of one type formed by both euspermatozoa and paraspermatozoa (3).

2. *Acrosome shape*: straight (0); twisted or corkscrew-shaped (1).

3. *Acrosome slenderness*: length/width ratio less than 8 (0); greater than 8 (1). The slenderness and absolute length of the acrosome vary among species; by presenting the states as ratios the influence of absolute length is eliminated.

4. *Acrosome vesicle withdrawal*: ratio acrosome vesicle length/length of portion withdrawn into acrosome tube considerably greater than 2 (0); less than 2 (1). We accept the assumption [40] that an acrosome vesicle external to the acrosome tube is plesiomorphic. The ratio eliminates the influence of absolute length.

5. *Acrosome tube*: thick-walled throughout (0); at least in part thin-walled (1).

6. *Secondary acrosome tube*: present (0); much reduced or absent (1).

7. *Acrosome rod (perforatorium)*: protuberant (anterior tip outside acrosome tube) (0); not protuberant (wholly located inside acrosome tube) (1).

8. *Shape of nucleus, anterior portion*: straight (0); twisted (1); corkscrew-shaped or flanged (2). Terminology according to FERRAGUTI *et al.* [24].

9. *Shape of nucleus, posterior portion*: straight (0); twisted (1). Nuclear shape is described as two characters, since different combinations of anterior and posterior shapes are present among tubificid spermatozoa.

10. *Number of mitochondria*: four or five (0); less than four (1). Four or five is a common number of mitochondria in "primitive spermatozoa" [26, 40].

11. *Mitochondrial shape*: straight and roundish-to-oval, length/width ratio not greater than about 1.5 (0); spiral and elongate, length/width ratio considerably greater than 1.5 (1).

12. *Central axonemal apparatus*: with prominent central sheath throughout flagellum (0); with tetragon fibres throughout flagellum (1); with prominent central sheath in anterior portion of flagellum, but tetragon fibres in posterior portion of flagellum (2). See [21].

Morphological characters.

13. *Hair setae*: present (0); absent (1). With reference to the condition in the outgroup Phreodrilidae, possession of hair setae has been interpreted as a plesiomorphic trait in the Tubificidae [15].

14. *Penial setae*: absent (0); present (1).

15. *Coelomocytes*: absent or few (0); numerous (1).

16. *Alimentary system*: present, body wall without symbiotic bacteria (0); absent, body wall with symbiotic bacteria (1).

17. *Oesophagus (in segment IX)*: unmodified (0); modified, either bearing a pair of diverticula, or dilated with reticulate blood plexus (1).

18. *Prostate glands*: diffuse (0); solid, pedunculate, one per atrium (1); lobed, broadly attached, one per atrium (2); solid, generally pedunculate, two per atrium (3); absent (4).

19. *Ciliation of atrial epithelium*: weak (cilia restricted to particular, ciliated cells) or absent (0); dense (atria heavily ciliated throughout) (1). In *Smithsonidrilus*, the atrial ampullae are ciliated, whereas the atrial ducts lack cilia; thus this character is coded as (0) for *Smithsonidrilus*.

20. *Penes*: poorly developed or absent (0); well developed, pendent within deep penial sacs (1).

Results of parsimony analyses

Analysis using only morphological characters (Fig. 3a). This analysis resulted in a single, most parsimonious, tree with 14 steps and a consistency index (CI) of 0.786. It suggests that Rhyacodrilinae, Phallogodrilinae and Limnodriloidinae are monophyletic taxa, but *Clitellio* appears

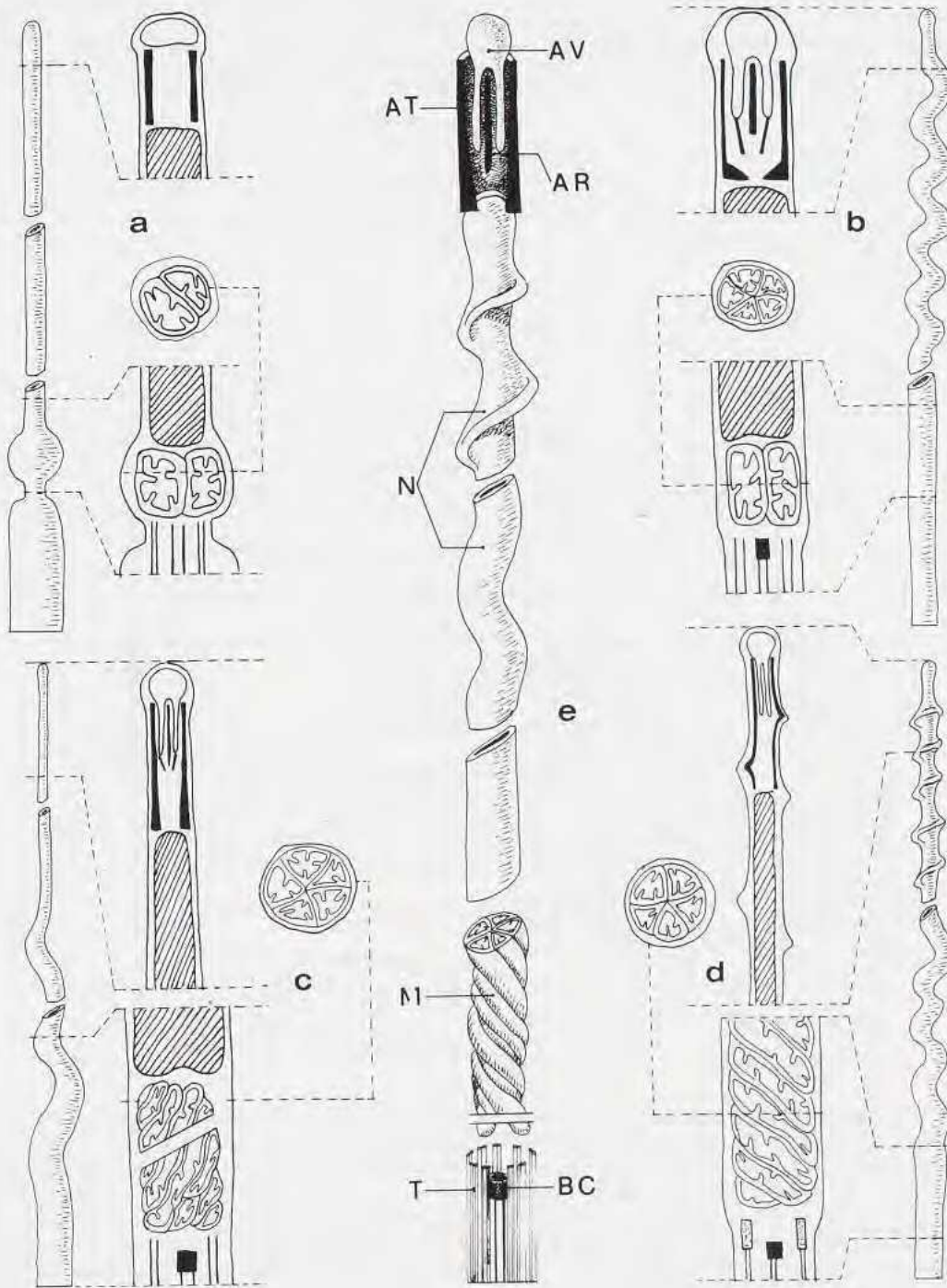


FIG. 2. — Schematic drawings of some tubificid sperm models. **a-b:** *Smithsonidrilus hummelincki*, paraspermatozoon (a) and euspermatozoon (b). **c:** *Heterodrilus pentcheffi*, euspermatozoon. **d:** *Coralliodrilus rugosus*, euspermatozoon. **e:** *Pectinodrilus molestus*, euspermatozoon. The plasma membrane has been omitted from the acrosome, mitochondria and tail of the *Pectinodrilus* spermatozoon (e), to show the internal structures. For abbreviations, see Fig. 1.

as the sister taxon of the Phallodrilinae rather than a member of the Tubificinae. The position of *Clitellio* is determined by its lack of prostate glands (state 4 of character 18), which is shared by *Coralliodrilus*, and the dense ciliation of its atria (character 19). For *Clitellio*, the coding of the latter character is based on a recent light microscopical study of *C. arenarius* [29]. Most other tubificines (including *Tubifex* and *Tubificoides*) are still assumed to have few cilia (if any at all) in their atria [15].

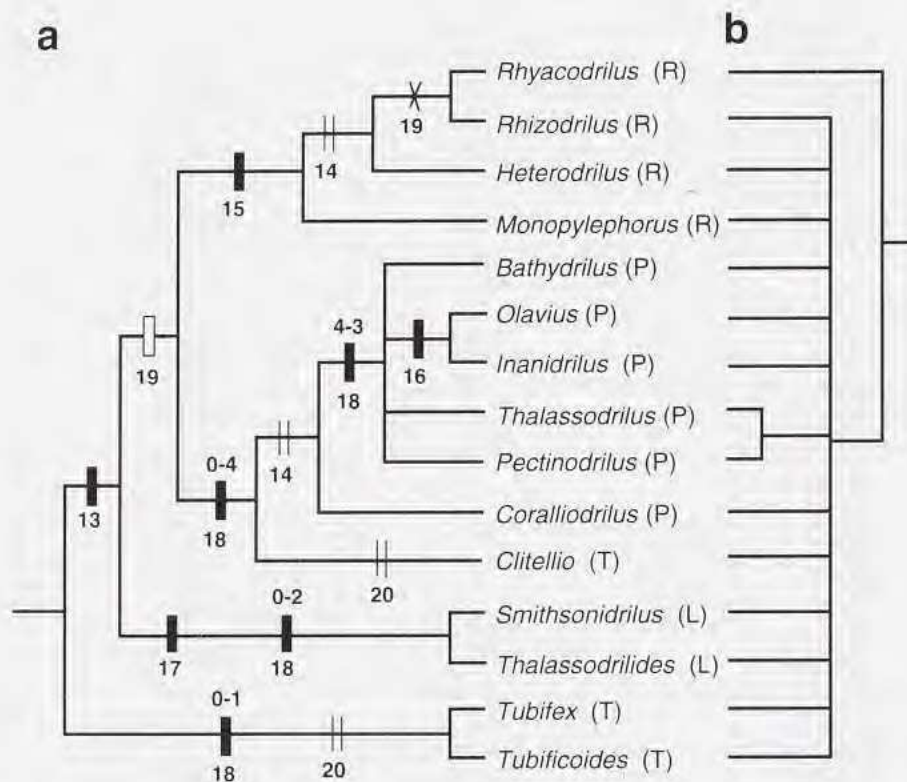


FIG. 3. — Results of parsimony analysis. **a**: The (one) most parsimonious tree for 15 taxa of Tubificidae, based on eight morphological characters (13-20). Length 14 steps; consistency index 0.786; retention index 0.893. Rooting at an hypothetical ancestor (see text). Subfamilial positions of taxa indicated in parentheses (L, Limnodriloidinae; P, Phallodrilinae; R, Rhyacodrilinae; T, Tubificinae). Numbers above symbols for multistate character refer to transformations of character states (e.g., 0-1 means "going from state 0 to state 1"). *Filled rectangle*, (unique) autapomorphy; *open rectangle*, autapomorphy that is followed by reversal further up the tree; *two parallel lines*, convergence; *cross*, reversal. **b**: Strict consensus tree of 45 equally parsimonious trees for 15 taxa of Tubificidae, based on spermatozoal characters (1-12).

Analysis using only spermatozoal characters (Fig. 3b). These characters yielded 45 equally parsimonious trees, all with 32 steps and a CI of 0.500. The congruence between these trees was very low, i.e., the spermatozoal characters proved more homoplastic than the morphological

characters. The only unequivocal suggestions from the sperm data are (1) that *Rhyacodrilus* has the most plesiomorphic spermatozoa known among the Tubificidae, and (2) that *Thalassodrilus* and *Coralliodrilus* are very closely related.

Analysis using both sets of characters (Fig. 4). When the spermatozoal and morphological characters were combined, the stability in tree topology increased considerably, although the CI (0.519) was barely greater than that of the trees based on only spermatozoal characters. The combined set gave three equally parsimonious trees, all with 52 steps, of which 36 emanate from the spermatozoal, 16 from the morphological characters. The trees differ only in the branching pattern of some phallodrilines, otherwise they all support (1) monophyly of each of Phallodrilinae, Limnodriloidinae and Tubificinae, (2) that the two latter are sister taxa, and (3) that the Phallodrilinae are cladistic members of the (otherwise paraphyletic) Rhyacodrilinae.

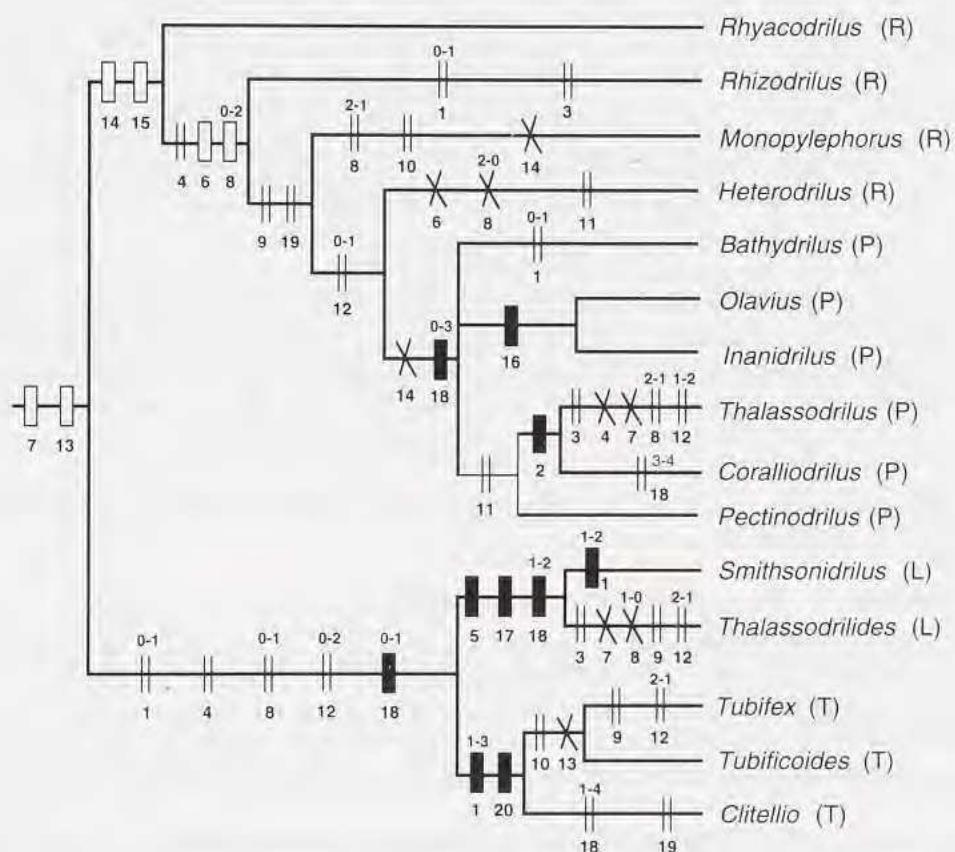


FIG. 4. — One of three equally parsimonious trees for 15 taxa of Tubificidae, based on twelve spermatozoal (1-12) and eight morphological characters (13-20). Length 52 steps; consistency index 0.519; retention index 0.638. Rooting at an hypothetical ancestor (see text). Branches shared by all three trees are indicated by bold lines; with another optimization of character 11, the group consisting of *Thalassodrilus* + *Coralliodrilus* and *Pectinodrilus* collapses. For other explanations, see Fig. 3.

DISCUSSION

The results of the parsimony analyses

The parsimony analyses revealed features of the character sets that were somewhat unexpected. The larger (spermatozoal) set could have been expected to yield the more stable trees, simply because on average there could be more character states to support each node in the trees. However, the poor congruence within the spermatozoal set made the morphological characters superior in terms of stabilizing tree topology.

With the two sets of characters combined, there is a trade-off between the congruence of the morphological data and the homoplasy of the spermatozoal data. Some traits of the tree based on morphology (Fig. 3a) are retained in the three trees based on all characters (Fig. 4), but there are also some changes. *Clitellio* now returns to the "tubificine" clade where it is normally classified, as the spermatozoal apomorphies (characters 1, 4, 8, 12) shared by *Clitellio* and *Tubifex/Tubificoides* (and partly by the limnodriloidines) outnumber the morphological ones shared by *Clitellio* and the phallodriline genus *Coralliodrilus* (characters 18, 19). The trees of the combined data set neither contradict the view that the Phallodrilinae, Limnodriloidinae and Tubificinae are monophyletic, nor that the Rhyacodrilinae are paraphyletic [15], although it should be noted that only a few selected genera of each subfamily are included in this analysis.

Two different hypotheses of subfamilial relationships within the Tubificidae, both based on conventional, morphological, characters, have recently been published. ERSÉUS [15] regarded the Phallodrilinae as an advanced subgroup within the "rhyacodriline" clade (a clade which also appears to contain the family Naididae, and possibly also the Opistocystidae), and this clade as a sister group of the rest of the family (Limnodriloidinae, Tubificinae and Telmatodrilinae). None of this is contradicted by the present study using the combined character sets; although the Telmatodrilinae are excluded from the present study. In the hypothesis presented by BRINKHURST [3], the Phallodrilinae is separated from the rhyacodriline clade and instead proposed to be the sister group of Limnodriloidinae-Tubificinae-Telmatodrilinae. This difference emanates from the fact that BRINKHURST's analysis included most aquatic oligochaete families in the ingroup, whereas the study by ERSÉUS focused on the Tubificidae and Naididae, using Phreodrilidae as the outgroup. BRINKHURST'S study implies that presence of hair setae is apomorphic when occurring in tubificid taxa, whereas this state turns out as a plesiomorphy in the present study as well as in that by ERSÉUS [15].

The relevance of sperm ultrastructure in studies of tubificid phylogeny

Both this and earlier studies [22, 24] show that tubificid sperm exhibit great variation in the ultrastructural details, sperm type differentiation, and formation of spermatozeugmata. Interestingly, the spermatozoa of species belonging to the same genus (although so far only investigated for *Inanidrilus* and *Heterodrilus*) are virtually identical, which indicates that spermatozoal characters are useful for the recognition of at least some genera within the Tubificidae.

Some spermatozoal features seem to be unique (aut)apomorphies of groups of genera (see Fig. 4): the twisted acrosome (character 2) of *Thalassodrilus* and *Coralliodrilus*, the partly thin-walled acrosome tube (character 5) of *Smithsonidrilus* and *Thalassodrilides*, and the double-line type of spermatozeugmata (character 1, state 3) of *Tubifex*, *Tubificoides* and *Clitellio*. However, a majority of sperm traits appear homoplastic. Much of the convergence is probably linked with the adaptive significance of some character states. For instance, very slender acrosomes (character 3) may have evolved at least three times in the Tubificidae; they are interpreted as independent transformations for *Rhizodrilus*, *Thalassodrilus* and *Thalassodrilides* (Fig. 4). The twisting of the nucleus (characters 8-9) seems to be convergent too, but here the most parsimonious hypotheses also imply reversal for these characters for some taxa (*Monopylephorus*, *Heterodrilus*,

Thalassodrilus and *Thalassodrilides*). The number of mitochondria has probably been reduced (character 10) at least twice, independently, for *Monopylephorus* and *Tubifex/Tubificoides*.

The withdrawal of the acrosome rod (character 7) is possibly an autapomorphy of the Tubificidae, but then this has become secondarily protuberant in *Thalassodrilus* and *Thalassodrilides*. Although the two latter genera clearly belong to different subfamilies, the spermatozoa of them exhibit several (apparently convergent) similarities: very slender acrosome, secondarily reduced (?) twisting of nucleus, and secondarily(?) protuberant acrosome rod. It is ironic that the type species of *Thalassodrilides* (*Limnodriloides gurwitschi* Hrabe) was once placed, for superficial morphological reasons, in *Thalassodrilus*; hence the name *Thalassodrilides* [see 4].

To conclude, tubificid spermatozoa provide a whole set of new ultrastructural characters, which are at least partly useful for phylogenetic assessments and, consequently, classification of taxa at different levels. However, the present study has shown that, in the Oligochaeta, the spermatozoal character patterns are complex and contain elements of convergence and probably also reversal, and therefore they should be used in tubificids only in combination with other information. Spermiocladistics, a term coined by JAMIESON [38], has proved useful for the reconstruction of phylogenies for many animal groups [37]. In many of the cases spermatozoal character patterns have been used to test, and indeed often to corroborate, hypotheses of phylogeny based on non-spermatozoal characters, or to propose new hypotheses of relationships. Thus, good congruence between spermatozoal and morphological data has been found at higher taxonomic levels in oligochaetes [34] and in the Clitellata, e.g. [21].

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